Biology

Biology fields

Okayama University *Year* 2004

Significant growth suppression of synovial sarcomas by the histone deacetylase inhibitor FK228 in vitro and in vivo

Tatsuo Ito[*] Mamoru Ouchida[†] Yuki Morimoto[‡]
Aki Yoshida[**] Yoshimi Jitsumori[††] Toshifumi Ozaki[‡‡]
Hiroshi Sonobe[§] Hajime Inoue[¶] Kenji Shimizu[‖]

[*]Okayama University
[†]Okayama University, ouchidam@md.okayama-u.ac.jp
[‡]Okayama University
[**]Okayama University
[††]Okayama University
[‡‡]Okayama University
[§]National Fukuyama Hospital
[¶]Okayama University
[‖]Okayama University

Significant growth suppression of synovial sarcomas by the histone deacetylase inhibitor FK228 *in vitro* and *in vivo*

Tatsuo Ito[1], Mamoru Ouchida[2,] *, Yuki Morimoto[1], Aki Yoshida[1], Yoshimi Jitsumori[2], Toshifumi Ozaki[1], Hiroshi Sonobe[3], Hajime Inoue[1], and Kenji Shimizu[2]

[1]Science of Functional Recovery and Reconstruction, Graduate School of Medicine and Dentistry, Okayama University, Shikata-cho 2-5-1, Okayama 700-8558, Japan

[2]Department of Molecular Genetics, Graduate School of Medicine and Dentistry, Okayama University, Shikata-cho 2-5-1, Okayama 700-8558, Japan

[3]Department of Laboratory Medicine and Pathology, National Fukuyama Hospital, Okinogami 4-14-17, Fukuyama, Hiroshima 720-8520, Japan

Subject area: Molecular and cell biology

*** Corresponding Author:** Dr. Mamoru Ouchida, Department of Molecular Genetics, Graduate School of Medicine and Dentistry, Okayama University, Shikata-cho 2-5-1, Okayama 700-8558, Japan

TEL: +81-86-235-7379 FAX: +81-86-235-7383

E-mail: ouchidam@md.okayama-u.ac.jp (M. Ouchida)

ABSTRACT

About 97% of synovial sarcomas harbor the *SYT-SSX* fusion gene by chromosomal translocation. We found that the histone deacetylase (HDAC) inhibitor FK228 significantly suppressed the growth of synovial sarcoma cells as compared with that of osteosarcoma. The 50% growth inhibition IC50 value we obtained for FK228 was 0.02-0.2 nM, and it indicates that its suppression effect on synovial sarcoma cells is the highest of any of the HDAC inhibitors yet reported. It was not likely that the growth suppression of FK228 depends on the doubling time of these cells. Introduction of *SYT-SSX* cDNA into HEK293 cells enhanced the sensitivity of the cells for FK228. Immunostaining of the FK228-treated cells using an anti-acetyl-histone H3 antibody showed that FK228 inhibits deacetylation of histone. In a mice assay, the growth of synovial sarcoma cells was markedly inhibited by FK228 treatment, and the invasion of tumors into surrounding tissues was suppressed. These results suggest that FK228 may be useful in developing therapeutic strategies to treat synovial sarcoma.

Key Words: histone deacetylase inhibitor, synovial sarcoma, growth inhibition, *in vivo.*

1. Introduction

Synovial sarcomas are soft tissue tumors, occurring at any age but affecting mainly young adults. They are considered high-grade sarcomas because they lead to death in at least 25% of patients within the first five years following diagnosis, despite advances in treatment [1]. These sarcomas are characterized cytogenetically by the presence of a chromosomal translocation t(X;18)(p11.2;q11.2), where the *SYT* gene on chromosome 18 is translocated to one of the closely related *SSX* family genes on chromosome X [2]. The SYT protein is associated with a chromatin remodeling factor hBRM/hSNF2 alpha protein [3], a transcriptional coactivator p300 protein [4], and a transcriptional factor AF10 [5]. Functional studies have shown that the acidic C-terminus of SSX carries a repressor domain [3, 6, 7]. SSX proteins have been reported to show colocalization with several members of the Polycomb group proteins which are associated with transcriptional repression [7]. Splicing isoform of the SYT-SSX fusion protein was reported to accelerate the transcriptional activity and cell proliferation [8].

Recently, we found that SYT proteins interact with mSin3A, a component of histone deacetylase (HDAC) complex, and reported that HDAC activity may be associated with

the tumorigenesis of synovial sarcomas [9]. In the present study, by using an HDAC-specific inhibitor FK228 (depsipeptide or FR901228) which was isolated from a culture broth of Chromobacterium violaceum [10, 11] and has been reported to be effective for treating malignant T-cell lymphomas [12], we examined the effect of HDAC inhibitor on synovial sarcoma cells with the chromosomal translocation t(X;18)(p11·2;q11·2). We found that the histone deacetylase inhibitor FK228 significantly suppressed the growth of synovial sarcoma cells as compared with that of osteosarcoma.

2. Materials and methods

2.1. Materials

Histone deacetylase inhibitor FK228 was provided by the Fujisawa Pharmaceutical Co. (Osaka, Japan). FK228 was dissolved in 0.1% ethanol for *in vitro* experiments, and dissolved in 10% polyoxyethylene-hydrogenated castor oil (Wako Pure Chemical Industries, Osaka, Japan) and diluted with 0.9% NaCl solution for *in vivo* experiments.

2.2. Cell lines

Synovial sarcoma cell lines HS-SY-2, YaFuSS and SYO-1 were provided by Dr. H. Sonobe (National Fukuyama Hospital, Hiroshima, Japan), Dr. J. Toguchida (Institute for Frontier Medical Sciences, Kyoto University, Japan) and Dr. A. Kawai (National Cancer Center, Tokyo, Japan), respectively. Osteosarcoma cell line, Saos2, and human normal embryonic kidney cell line, HEK293, were purchased from American Type Culture Collection.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Synthesis of cDNA was performed using ReverTra Ace (Toyobo, Tokyo, Japan). Identification of *SYT-SSX* cDNA was performed by PCR with *SYT* forward primer 5'-CAA CAG CAA GAT GCA TAC CA and *SSX1* reverse primer 5'-ACA CTC CCT TCG AAT CAT TTT CG or *SSX2* reverse primer 5'-GCA CTT CCT CCG AAT CAT TTC as described in a previous paper [13]. The amplified products were confirmed by DNA sequencing with an ABI3100 sequencer

(Applied Biosystems, Foster City, CA, USA).

2.4. Tumor growth assay in vitro

All cell lines were split into 96-well plates (10^3 cells/well), and cultured with 0.001-100 nM of histone deacetylase inhibitor FK228. After exposure to FK228 for 24, 48 and 72 hrs, cell viability was determined in four individual experiments by MTT assay kit (Chemicon International Inc., Temecula, CA) according to the Manufacturer's instructions. IC50 was determined by nonlinear regression analysis applying the inhibition data to the dose-response curve.

2.5. Transfection

Human embryonic kidney 293 cells were transfected with pCMV-SYT-SSX1 and pCMV-SYT-SSX2, which contain the cDNAs amplified by RT-PCR [9], by Effectene reagent (Qiagen, Hilden, Germany), and selected with 400 μg /ml of G418 (Sigma, St. Louis, MO, USA) for 2 weeks.

2.6. Tumor growth assay in vivo

Animals were treated according to the animal experimental guidelines of Okayama University. Twenty male mice (BALB/c nu/nu) were inoculated subcutaneously with SYO-1 synovial sarcoma cells (10^5 cells/mouse). Injection of FK228 (50 μl) into the veins of the 20 mice was started 10 days after subcutaneous inoculation of SYO-1 cells, and performed 3 times every 4 days. Body weight was measured every 4 days. Tumor volume was measured every 4 days for two right-angled diameters with slide calipers, and was calculated using the following formula: tumor volume $= 1/6\,\pi\,[(d1 \times d2)^{3/2}]$ (where d1 and d2 are the two perpendicular diameters)[14]. Using another set of the inoculated 20 mice, tumors were resected at day 20 after subcutaneous inoculation of SYO-1 cells, and tumor weight was measured after the invaded internal organs were removed from them as well as possible.

The relationship between the tumor weight and FK228-treatment was statistically analyzed by Student's t-test.

2.7. Histopathology

Tumors were fixed overnight in 10% (v/v) formaldehyde/0.9% NaCl solution before paraffin embedding and routine sectioning. Three representative hematoxylin-eosin stained sections were examined for each tumor. Specimens on slides were incubated with anti-histone H3 (1:200) (Cell Signaling Technology, Beverly, MA, USA) or anti-acetylated histone H3 antibody (1:200) (Cell Signaling Technology) for 1 hr at room temperature. Immunoreactive signals were visualized by FITC-labeled rabbit IgG or by using diaminobenzidine.

3. RESULTS

We confirmed the expression of fusion genes in synovial sarcoma cell lines by RT-PCR. HS-SY-2 and YaFuSS expressed the *SYT-SSX1* gene, and SYO-1 expressed the *SYT-SSX2* gene (Figure 1, lanes 1-3), suggesting that these cell lines are derived from synovial sarcomas. As a control, Saos2 osteosarcoma cell line was used and shown no *SYT-SSX* fusion genes (Figure 1, lanes 4). Next, we examined the effect of FK228 on the growth of these cells. Most notably, a significant growth inhibition was observed in the synovial

sarcoma cells as compared with that of Saos2 (Figures. 2A-D and 3). The concentration of FK228 at 50% growth inhibition (IC50) was 0.2 nM in HS-SY-2, 0.02 nM in YaFuSS and 0.03 nM in SYO-1 at 48 hr after treatment (Table 1), while osteosarcoma cell line, Saos2, showed 9.33 nM of IC50. Doubling time of these cells was measured, but it is not likely that the growth suppression of FK228 depends on the doubling time (Table 1). SYO-1 cells carrying the *SYT-SSX2* gene showed more sensitive growth inhibition to FK288 at least at 24 hr than HS-SY-2 and YaFuSS both of which have the *SYT-SSX1* gene. To examine whether the SYT-SSX fusion proteins affect on the growth suppression by FK228, the *SYT-SSX* cDNA expression vectors were transfected into HEK293 cells. G418-resistant clones were gathered, and the *SYT-SSX* gene expression was confirmed by RT-PCR (Figure 1, lanes 5-7). These transformants expressing *SYT-SSX* gene showed an enhanced sensitivity to FK228 when compared to that of the cells bearing empty vector (Figure 2E). Growth of HEK293 cells carrying the *SYT-SSX2* gene was suppressed at lower concentration of FK228 than that of the *SYT-SSX1* transformants was. Immunostaining of the FK228-treated SYO-1 cells using an anti-acetyl-histone H3 antibody showed the nuclear localization of the acetylated histone, suggesting that FK228

inhibits deacetylation of histone (Figure 4).

The SYO-1 cell line was used to examine the tumor suppression effect of FK228 on synovial sarcoma cells *in vivo*, because the cell line is known to grow well in mice xenografts. Mice treated with FK228 (1.6 and 3.2 mg/kg mouse) showed significantly reduced growth of SYO-1 cells when compared to those treated with the vehicle (Figures 5A and 6A). The tumors resected from FK228-treated mice exhibited significantly weight loss more than those from mice treated with the vehicle (Figure 5B). Intra-abdominal invasion of the tumor tissues was observed in most mice in the vehicle-treated group, and the internal organs of the back/peritoneal region, including the spine and kidney, were involved in the tumors (Figure 6B). However, intra-abdominal invasion was not observed in any of the FK228-treated mice (Figure 6C). HE staining of tumors revealed that the tumors of the vehicle-treated group were of a high density, while those of the FK228-treated group were of a comparatively low density (Figure 6D-F). Epithelioid-like spindle cells and round cells were observed in vehicle-treated group, while spindle cells were absent in the tumors from the FK228-treated group. In the tumors from FK228-treated group, infiltration of lymphocytes was frequently detected, and a high rate

of necrosis was observed (data not shown). Immunohistochemical studies using anti-acetylated histone antibody showed the presence of acetylated histone in tumor tissues of the FK228-treated group (Figure 6H), suggesting that this drug inhibits deacetylation of histone in tumors of FK228-treated mice. The body weight of the mice treated with FK228 showed a transient decrease upon treatment with the drug (Figure 5C), suggesting that it might produce some side effects.

4. Discussion

Recently, we reported that SYT proteins interact with mSin3A, a component of HDAC complex [9], which suggests that HDAC activity may be associated with the tumorigenesis and malignancy of synovial sarcoma. Therefore, by using HDAC inhibitor FK228, we examined the effect of the HDAC inhibitor on growth of synovial sarcoma. Interestingly, a significant growth inhibition was observed in the synovial sarcoma cells as compared with that of Saos2. A recent study on acute myelogenous leukemias revealed that the AML1-ETO fusion protein disrupts normal hematopoiesis by blocking trans-activation of AML1 target genes through recruitment of HDAC1 by ETO [15], in

addition to reports on the anti-tumor effects of HDAC inhibitors on various cancer cells [16]. The LC50 (50% cytotoxicity) of FK228 for the acute myelogeneous leukemia cells was 4.6 nM at 48 hrs [17], while the IC50 value of FK228 in our results was 0.02-0.2 nM in synovial sarcoma cells at 48 hrs after treatment. This is the lowest concentration yet reported for any of the known HDAC inhibitors. Our results of *SYT-SSX* cDNA-transfection experiment suggest that SYT-SSX fusion protein may associate with the growth suppression by FK228. The relation between the SYT protein and histone acetylation/deacetylation has been also implied in studies of histone acetyltransferase p300 [4] and mSin3A [9]. Our results showed that the cells carrying the *SYT-SSX2* gene are more sensitive to FK228 than the cells carrying the *SYT-SSX1* gene. It has been reported that the type of the *SYT-SSX* fusion gene influences the clinical outcome [18, 19], and the phenomenon we found may be associated with the mechanism by which the clinical outcome is affected. But further studies with lots of cell lines will be required to clarify the unsolved issues. High viability of SYO-1 cells treated with FK228 at a concentration of 0.001 nM was reproducibly observed (Figure 2C). SYO-1 cells may be still affected by low concentration (0.001 nM) of FK228, but may be able to overcome the

stress by cellular defense system like degradation or export of the reagent at lower concentration, leading to the stimulation of the cell growth by unknown mechanism. The IC50 of osteosarcoma cell line, Saos2, was reported to be 2.6 nM at 72 hr [20], and it was 9.33 nM at 48 hr in our results. There is a tendency that the sensitivity of Saos2 for FK228 in our experiment is lower than that of their report, but it might be caused by different subtypes of culture cells.

In the FK228-treated mice, synovial sarcoma cells were significantly reduced in size and did not show invasion into other organs, while weight reduction was transiently observed in mice treated with a high concentration of FK228. A phase1 study with FK228 showed that dose-limited toxicity caused fatigue, nausea, vomiting, and transient thrombocytopenia and neutropenia [21]. Moreover, reversible electrocardiogram (ECG) changes with ST/T wave flattening were regularly observed in 37 malignant tumor patients [21]. Although FK228 is currently expected to be used as an universal anti-neoplastic agent , it may be necessary to use the drug in low concentrations to avoid producing some of the side effects mentioned here.

SYT-SSX fusion gene has been detected in nearly 97% cases of synovial sarcomas [22].

At present, the 5-year overall survival rates for synovial sarcomas are 53% for the *SYT-SSX1* type and 73% for the *SYT-SSX2* type [23]. Our findings suggest that FK228 may be useful in developing therapeutic strategies to treat this form of sarcoma. Identification of molecule targets of FK228 and clinical trials of FK228 are necessary to find an effective cure for malignant synovial sarcomas, and to gain insight into the mechanisms of tumorigenesis in synovial sarcomas.

Acknowledgements

We are grateful to the Fujisawa Pharmaceutical Co. (Osaka, Japan) for kindly providing FK228.

References

[1] J.J. Lewis, C.R. Antonescu, D.H. Leung, D. Blumberg, J.H. Healey, J.M. Woodruff, M.F. Brennan. Synovial sarcoma: a multivariate analysis of prognostic factors in 112 patients with primary localized tumors of the extremity, J. Clin. Oncol. 10 (2000) 2087-2094.

[2] C. Turc-Carel, P. Dal Cin, J. Limon, F. Li, A.A. Sandberg. Translocation X;18 in synovial sarcoma. Cancer Genet. Cytogenet. 23 (1986) 93.

[3] C. Thaete, D. Brett, P. Monaghan, S. Whitehouse, G. Rennie, E. Rayner, C.S. Cooper, G. Goodwin. Functional domains of the *SYT* and *SYT-SSX* synovial sarcoma translocation proteins and co-localization with the SNF protein BRM in the nucleus, Hum. Mol. Genet. 8 (1999) 585-591.

[4] J.E. Eid, A.L. Kung, R. Scully, D.M. Livingston. p300 interacts with the nuclear proto-oncoprotein SYT as part of the active control of cell adhesion, Cell 102 (2000) 839-848.

[5] D.R. de Bruijn, N.R. dos Santos, J. Thijssen, M. Balemans, S. Debernardi, B. Linder, B.D. Young, A. Geurts van Kessel. The synovial sarcoma associated protein SYT

interacts with the acute leukemia associated protein AF10, Oncogene 20 (2001) 3281-3289.

[6] D. Brett, S. Whitehouse, P. Antonson, J. Shipley, C. Cooper, G. Goodwin. The SYT protein involved in the t(X;18) synovial sarcoma translocation is a transcriptional activator localized in nuclear bodies, Hum. Mol. Genet. 6 (1997) 1559-1564.

[7] F.L. Lim, M. Soulez, D. Koczan, H.J. Thiesen, J.C. Knight. A KRAB-related domain and a novel transcription repression domain in proteins encoded by *SSX* genes that are disrupted in human sarcomas, Oncogene 17 (1998) 2013-2018.

[8] Y. Morimoto, M. Ouchida, T. Ozaki, A. Kawai, T. Ito, A. Yoshida, H. Inoue, K. Shimizu. Splicing isoform of SYT-SSX fusion protein accelerates transcriptional activity and cell proliferation, Cancer Lett. 199 (2003) 35-43.

[9] T. Ito, M. Ouchida, S. Ito, Y. Jitsumori, Y. Morimoto, T. Ozaki, A. Kawai, H. Inoue, K. Shimizu. SYT, a partner of SYT-SSX oncoprotein in synovial sarcomas, interacts with mSin3A, a component of histone deacetylase complex, Lab Invest. 84 (2004) 1484-1490.

[10] H. Ueda, H. Nakajima, Y. Hori, T. Fujita, M. Nishimura, T. Goto, M. Okuhara. FR901228, a novel antitumor bicyclic depsipeptide produced by Chromobacterium

violaceum No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity, J. Antibiot. 47 (1994) 301-310.

[11] H. Nakajima, Y.B. Kim, H. Terano, M. Yoshida, S. Horinouchi. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor, Exp. Cell Res. 241 (1998) 126-133.

[12] R.L. Piekarz, R. Robey, V. Sandor, S. Bakke, W.H. Wilson, L. Dahmoush, D.M. Kingma, M.L. Turner, R. Altemus, S.E. Bates. Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and coetaneous T-cell lymphoma: a case report, Blood 98 (2001) 2865-2868.

[13] N. Naito, A. Kawai, M. Ouchida, T. Dan'ura, Y. Morimoto, T. Ozaki, K. Shimizu, H. Inoue. A reverse transcriptase-polymerase chain reaction assay in the diagnosis of soft tissue sarcomas, Cancer 89 (2000) 1992-1998.

[14] D.M. Vigushin, S. Ali, P.E. Pace, N. Mirsaidi, K. Ito, I. Adcock, R.C. Coombes. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo, Clin. Cancer Res. 7 (2001) 971-976.

[16] W.K. Kelly, O.A. O'Connor, P.A. Marks. Histone deacetylase inhibitors: from target

to clinical trials, Expert Opin. Investig. Drugs 11 (2002) 1695-1713.

[15] V. Gelmetti, J. Zhang, M. Fanelli, S. Minucci, P.G. Pelicci, M.A. Lazar. Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO, Mol. Cell Biol. 18 (1998) 7185-7191.

[17] M.I. Klisovic, E.A. Maghraby, M.R. Parthun, M. Guimond, A.R. Sklenar, S.P. Whitman, K.K. Chan, T. Murphy, J. Anon, K.J. Archer, L.J. Rush, C. Plass, M.R. Grever, J.C. Byrd, G. Marcucci. Depsipeptide (FR901228) promotes histone acetylation, gene transcription, apoptosis and its activity is enhanced by DNA methyltransferase inhibitors in AML1/ETO-positive leukemic cells, Leukemia 17 (2003) 350-358.

[18] A. Kawai, J. Woodruff, J.H. Healey, M.F. Brennan, C.R. Antonescu, M. Ladanyi. SYT-SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma, N. Engl. J. Med. 338 (1998) 153-160.

[19] G. Nilsson, B. Skytting, Y. Xie, B. Brodin, R. Perfekt, N. Mandahl, J. Lundeberg, M. Uhlen, O. Larsson. The SYT-SSX1 variant of synovial sarcoma is associated with a high rate of tumor cell proliferation and poor clinical outcome, Cancer Res. 59 (1999) 3180-3184.

[20] T. Imai, S. Adachi, K. Nishijo, M. Ohgushi, M. Okada, T. Yasumi, K. Watanabe, R. Nishikomori, T. Nakayama, S. Yonehara, J. Toguchida, T. Nakahata. FR901228 induces tumor regression associated with induction of Fas ligand and activation of Fas signaling in human osteosarcoma cells, Oncogene 22 (2003) 9231-9242.

[21] V. Sandor, B. Susan, R.W. Robey, M.H. Kang, M.V. Blagosklonny, J. Bender, R. Brooks, R.L. Piekarz, E. Tucker, W.D. Figg, K.K. Chan, B. Goldspiel, A.T. Fojo, S.P. Blacerzak, S.E. Bates. Phase 1 Trial of the Histone Deacetylase Inhibitor, Depsipeptide (FR 901228, NSC 630176), in Patients with Refractory Neoplasms, Clin. Cancer Res. 8 (2002) 718-728.

[22] N.R. dos Santos, D.R. de Bruijn, A. Geurts van Kessel. Molecular mechanisms underlying human synovial sarcoma development, Genes Chromosomes Cancer 30 (2001) 1-14.

[23] M. Ladanyi, C.R. Antonescu, D.H. Leung, J.M. Woodruff, A. Kawai, J.H. Healey, M.F. Brennan, J.A. Bridge, J.R. Neff, F.G. Barr, J.D. Goldsmith, J.S. Brooks, J.R. Goldblum, S.Z. Ali SZ, J. Shipley, C.S. Cooper, C. Fisher, B. Skytting, O. Larsson. Impact of SYT-SSX fusion type on the clinical behavior of synovial sarcoma: a multi-institutional retrospective study of 243 patients, Cancer Res. 62 (2002) 135-140.

Figure legends

Figure 1

Detection of the *SYT-SSX* transcripts in cell lines. *SYT-SSX1* (331 bp) and *SYT-SSX2* (331 bp) cDNAs were detected by RT-PCR. M, DNA molecular weight marker; lane 1, SYO-1; lane 2, YaFuSS; lane 3, HS-SY-2; lane 4, Saos2; lane 5, HEK293 transfected with an empty vector; lane 6, HEK293 transfected with *SYT-SSX1* cDNA expression vector; lane 7, HEK293 transfected with *SYT-SSX2* cDNA expression vecter.

Figure 2

Effect of FK228 on the growth of cell lines. HS-SY-2 (A), YaFuSS (B), SYO-1 (C) and Saos2 (D) were measured using MTT assay at 24, 48 and 72 hr after addition of FK228 (0.001-100 nM). HEK293 cells bearing cDNA expression vector (E) were measured at 48 hr after addition of FK228. Viability was calculated by dividing the value of FK228-treated cells by the value of the cells treated with the vehicle (0.1% ethanol). Data are represented as the mean + s.d. of four individual experiments.

Figure 3

Effect of FK228 on the growth of cell lines. YaFuSS and Saos2 were cultured for 48 hr after addition of FK228 (0 - 100 nM) and observed with magnification of X100.

Figure 4

Inhibition of histone deacetylase by FK228. Immunostaining of SYO-1 cells is shown using acetyl-histone H3 antibody (A, C and E) and histone H3 antibody (B, D and F) after vehicle-treatment (A and B) and FK228-treatment of 0.1 nM (C and D) and 1 nM (E and F) for 3 hrs. Immunoreactive signals were visualized by FITC-labeled rabbit IgG.

Figure 5

Suppression of SYO-1 cells in mice by FK228. Injection of FK228 into the veins of 20 mice was started 10 days after subcutaneous inoculation of SYO-1 cells (10^5 cells/mouse), and performed 3 times every 4 days as shown by *arrows*. Tumor volume (A) and body weight (C) were measured every 4 days. Using another set of the inoculated 20 mice, tumor weight was measured by resecting at day 20 after subcutaneous inoculation of

SYO-1 cells (B). Vehicle, vehicle-treated mice; 1.6 mg/kg, FK228 (1.6 mg/kg)-treated mice; 3.2 mg/kg, FK228 (3.2 mg/kg)-treated mice; 3.2 mg/kg w/o tumor, FK228 (3.2 mg/kg)-treated mice without subcutaneous inoculation of SYO-1 cells. *P*-value is shown against vehicle-treated tumor weight. Data are represented as the mean + s.d.

Figure 6

Characterization of SYO-1 cells suppressed by FK228. (A) Mice treated with the vehicle (*left*) and with a concentration of 1.6 mg/kg of FK228 (*center*) and with a concentration of 3.2 mg/kg of FK228 (*right*). (B and C) Tumor cells in the mice treated with the vehicle (B) and 3.2 mg/kg of FK228 (C). (D-F) Hematoxylin-eosin staining of tumor tissues. (D) Tumor tissue treated with the vehicle. This tumor was composed of spindle and round cells with different cell sizes and atypical nuclei. (E and F) Tumor tissues treated with FK228 at a concentration of 1.6 mg/kg and 3.2 mg/kg, respectively. The living cells were mostly round cells, with few spindle cells remaining. (G-J) Immunohistochemical studies of acetylated histone H3. Tumors in mice treated with the vehicle (G and I) and a concentration of 3.2 mg/kg of FK228 (H and J) were immunostained with anti acetyl-histone H3 (G and H) and anti-histone H3 antibody (I and J) at day 22 after inoculation of SYO-1 cells into another set of mice. Immunoreactive signals were visualized with diaminobenzidine.

Figure 1

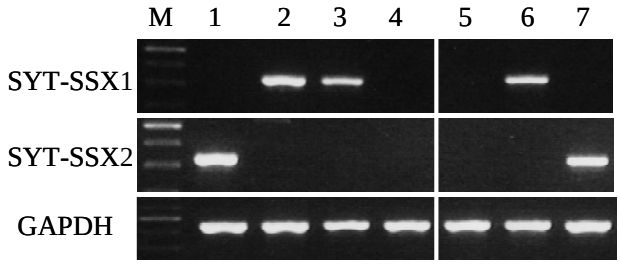

Figure 2

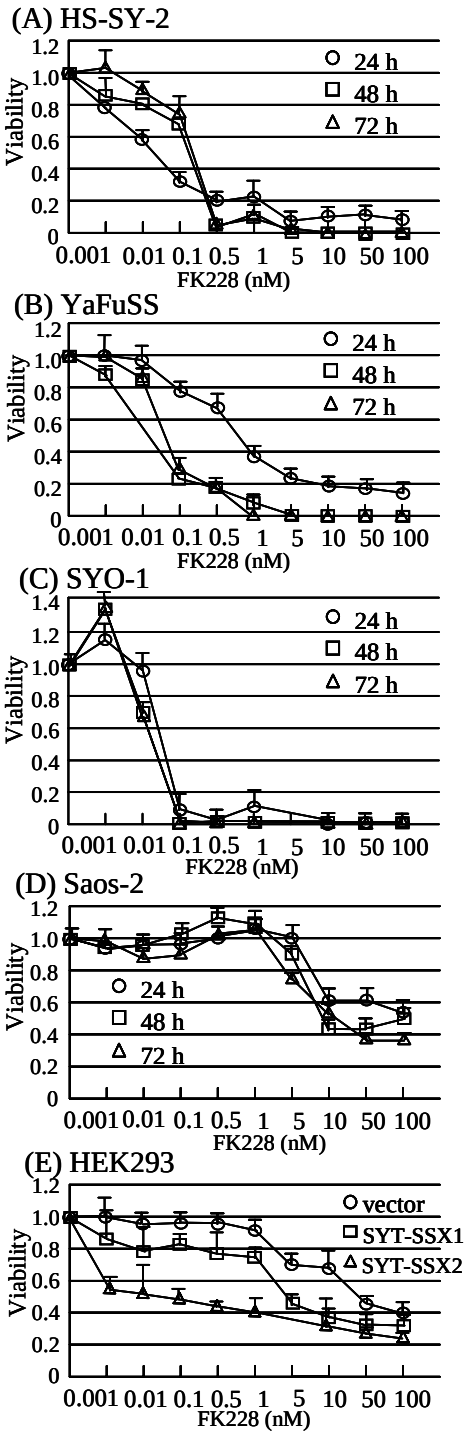
(A) HS-SY-2
Viability
1.2
1.0
0.8
0.6
0.4
0.2
0
24 h
48 h
72 h
0.001 0.01 0.1 0.5 1 5 10 50 100
FK228 (nM)
(B) YaFuSS
Viability
24 h
48 h
72 h
FK228 (nM)
(C) SYO-1
Viability
1.4
24 h
48 h
72 h
FK228 (nM)
(D) Saos-2
Viability
24 h
48 h
72 h
FK228 (nM)
(E) HEK293
Viability
vector
SYT-SSX1
SYT-SSX2
FK228 (nM)

Figure 3

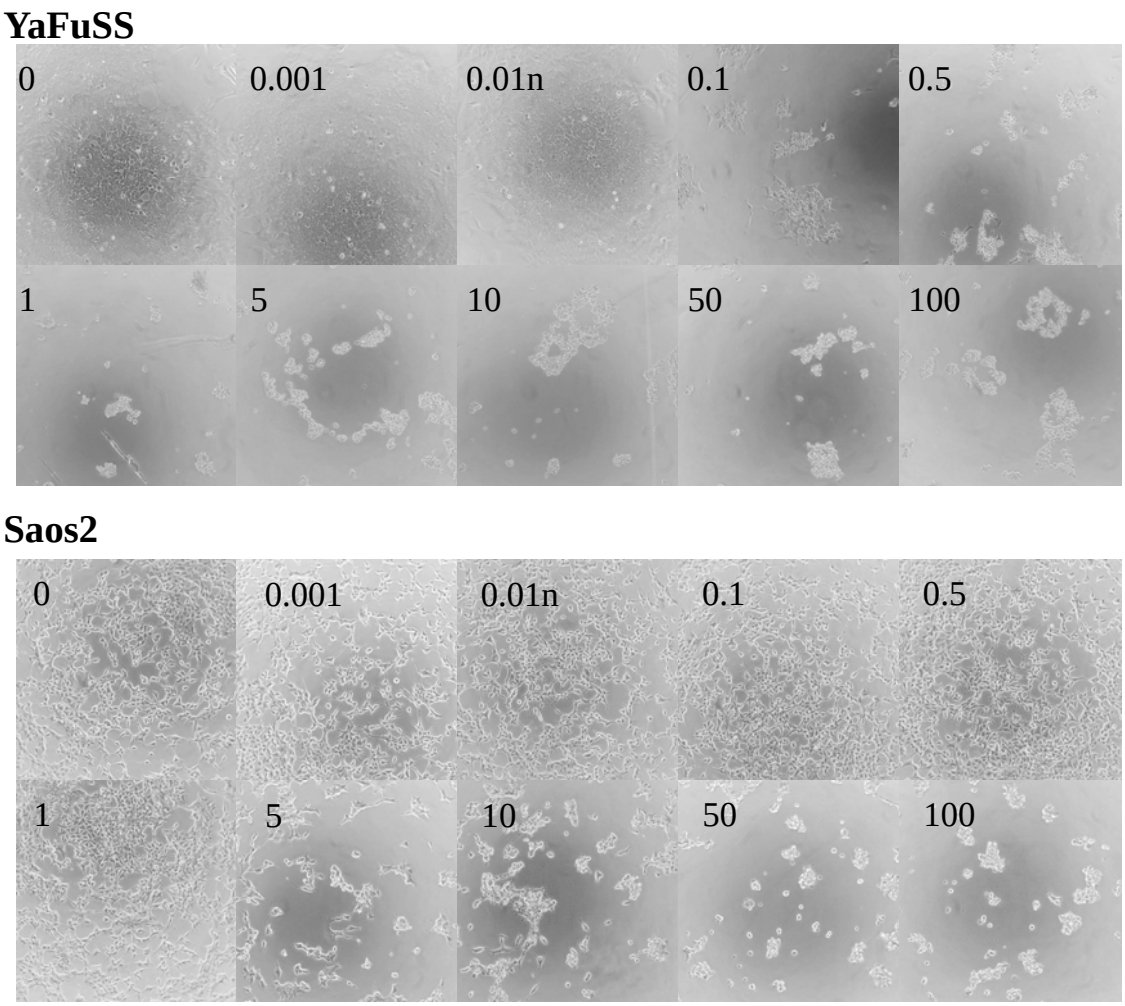
YaFuSS
0
0.001
0.01n
0.1
0.5
1
5
10
50
100
Saos2
0
0.001
0.01n
0.1
0.5
1
5
10
50
100

Figure 4

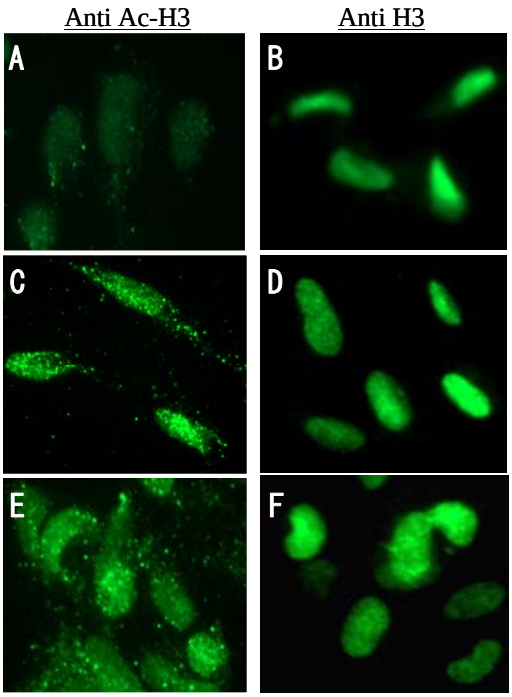

Figure 5

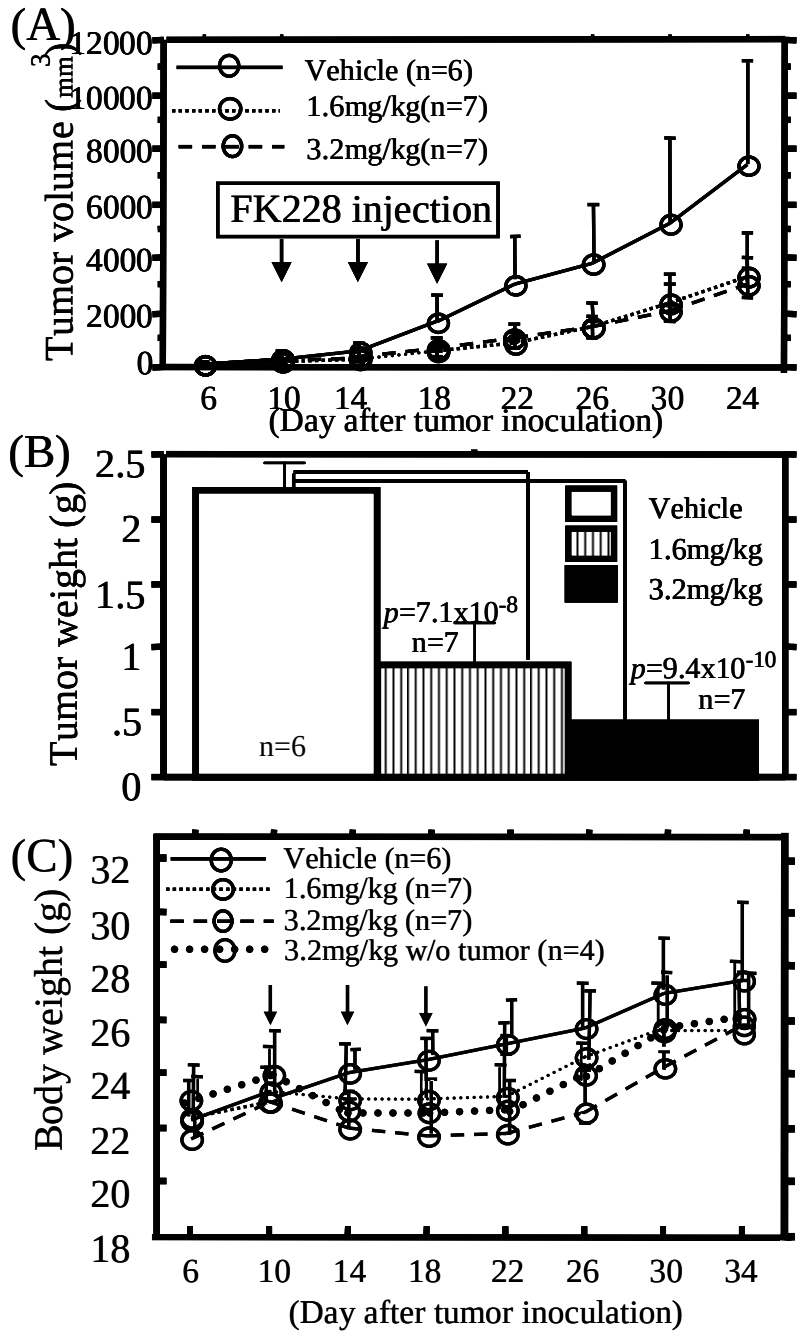
(A)
Tumor volume (mm3)
12000
10000
8000
6000
4000
2000
0
Vehicle (n=6)
1.6mg/kg(n=7)
3.2mg/kg(n=7)
FK228 injection
6
10
14
18
22
26
30
24
(Day after tumor inoculation)
(B)
Tumor weight (g)
2.5
2
1.5
1
.5
0
Vehicle
1.6mg/kg
3.2mg/kg
p=7.1x10-8
n=7
p=9.4x10-10
n=7
n=6
(C)
Body weight (g)
32
30
28
26
24
22
20
18
Vehicle (n=6)
1.6mg/kg (n=7)
3.2mg/kg (n=7)
3.2mg/kg w/o tumor (n=4)
6
10
14
18
22
26
30
34
(Day after tumor inoculation)

Figure 6

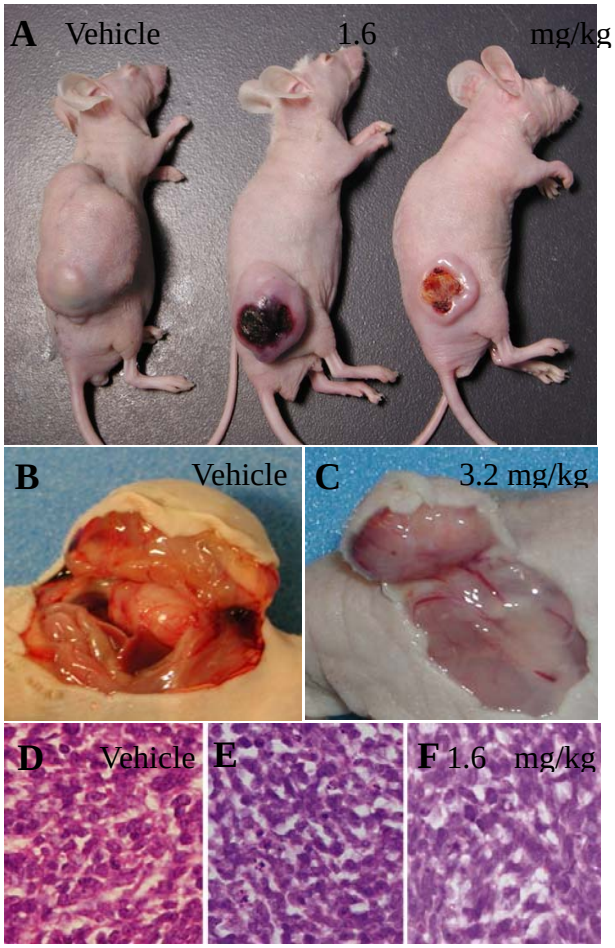

Table 1

IC50 of FK228 and doubling time of sarcoma cells

Cells	Fusion gene	IC50[1]	Doubling time (hr)
HS-SY2	SYT-SSX1	0.20	25.0
YaFuSS	SYT-SSX1	0.02	24.9
SYO-1	SYT-SSX2	0.03	18.6
Saos2	none	9.33	17.9

[1] IC50; 50% growth inhibition value (nM) at 48 hr after FK228 addition